BANKING ON TISSUES

Twenty years ago this July in Seveso, Italy, a reaction vessel in a chemical factory exploded, spewing a foul-smelling compound that caused birds to fall from the sky. Over the next month, 200 residents developed chloracne, indicating exposure to dioxin, says Neil Caporaso, a scientist in the Genetic Epidemiology Branch of the National Cancer Institute. All farm animals were slaughtered and the town was divided up into several zones based on level of exposure. In the most heavily exposed districts, the soil was dug up and carted away, and houses were razed. Fortunately, blood samples of residents had been banked, for although only the highest exposures could be measured at the time, now "we can classify the exposures that literally hundreds of people had, and tell what areas were hit," says James Pirkle, assistant director for science in the Division of Environmental Health Laboratory Sciences of the Centers for Disease Control (CDC). "Some areas were declared safe that actually were hit."

Studies based on these samples are beginning to reveal the relationship of dioxin exposure to various cancers, and researchers have been able to go back to exposed individuals and find out how strongly the compound has persisted in their bodies. "Much of our understanding about the persistence of dioxin in humans depends on this," says Caporaso.

"Banked specimens present a great resource for evaluating environmental and occupational health problems," says Paul A. Schulte, acting director of the Education and Information Division of the National Institute for Occupational Safety and Health. "Human tissue samples really [provide] a measure of dose," explains Ken Sexton, professor of environmental health at the University of Minnesota School of Public Health.

The Holy Grail of tissue banking is to relate levels of environmental contamination to health outcomes via doses—the main pieces of information one looks for in banked samples, says Jack A. Taylor, head of the Molecular and Genetic Epidemiology Group at the NIEHS. Unfortunately, samples are often banked without information on exposures, and cohorts are often not followed because it is difficult and expensive to do. Nonetheless, there is plenty of value in more narrowly designed studies.

Lately, a controversy over informed consent has flared, threatening to force researchers to obtain new consent from every individual in a cohort study every time something new is done with the banked materials. The issue "terrorizes me," says Taylor.

The controversy is particularly threatening to researchers who use tissue banks in one of their newest applications: research on the genetics of susceptibility to specific carcinogens. "If we don't solve this dilemma, no prospective cohort that we design now will be usable," says John Groopman, professor and chairperson of the Department of Environmental Health Sciences at the Johns Hopkins University School of Hygiene and Public Health of the issue of consent.

National Monitoring

The monitoring of exposures to dioxin at Seveso represents a quick response to an acute and obvious problem. There is also a need to track chemical contamination of human tissues on a more ongoing basis, according to the National Research Council (NRC). In the United States, more than 60,000 chemicals were

approved for commercial use in 1984. Of 3,400 pesticide ingredients, only about 10% had data available for a complete health hazard assessment, and more than 700 organic chemicals have been identified in U.S. drinking water.

Several federal programs have experimented with population-based monitoring of human tissue samples. The National Human Adipose Tissue Survey (NHATS), an EPA program, scrutinized mainly pesticide residues from 1967 to 1989, collecting about 12,000 tissue samples, mostly from cadavers. But NHATS was cited by the NRC for quality control and other problems and is virtually defunct. The National Health and Nutrition Examination Surveys (NHANES) of the National Center for Health Statistics collects blood periodically from a population-based sample of about 20,000 U.S. citizens, but the focus is mostly on nutrition.

Recently, several agencies including the EPA and the CDC have established a pilot population-based survey, the National Human Exposure Assessment Survey (NHEXAS), to monitor blood (50 ml per subject), urine, hair, and in some cases, fingernail, samples while also sampling the air, water, soil, food, and dust from inside the houses of subjects, says Sexton, who is helping to run the program. Subjects will answer a questionnaire concerning age, gender, occupation, type of housing, and indoor exposures to toxins from sources such as gas stoves and cigarettes. The survey will be conducted on 50 people in the Baltimore metropolitan area, 300–400 people in Arizona, and about 500 people in EPA Region 5 (which includes Indiana, Illinois, Michigan, Minnesota, Ohio, and Wisconsin), says Sexton. Each pilot area is run by a consortium of scientists who will choose what to sample from among metals, volatile organics, pesticides and PAHs. The same compounds will be sampled in human subjects and the environment.

According to Jerry Akland, project officer for NHEXAS, the project's success will be measured by how much the additional information reduces uncertainty in current exposure models. In other words, if the distribution of exposures is different from what current models predict, it will justify the cost of a national survey. Such a survey of about 5,000 people would be conducted with subjects selected through statistically-based sampling design. "This will give a pretty good sense of average exposures, and . . . over time, you can see the trends," says Sexton. "Almost without exception, we don't have that kind of information in this country."

What Tissue Banking Can Tell Us

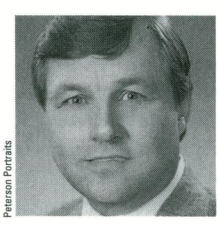
Despite the lack of a direct link of exposures to adverse health outcomes, a national monitoring study could be critically important. The data could "help identify new or previously unrecognized hazards . . . [and] establish trends in body burdens of toxicants that result from changes in manufacture, use, and disposal patterns, and thus monitor the results of programs intended to control specific chemical hazards," according to the NRC's 1991 study, *Monitoring Human Tissues for Toxic Substances.*

For example, roughly five years after the pesticide Mirex was used to kill fire ants, retrospective analysis of NHATS tissues from the period when it was used found contamination in human tissues. "Evidence of Mirex exposure . . . led to a more intensive survey of the general population in treated counties of the southeastern United States," according to a 1985 article in the *Journal of Toxicology and Environmental Health.*

Nationwide surveys are too broad to furnish definitive data on health effects, says John C. Bailar, III, chairperson of the Department of Health Studies at the University of Chicago. But they are very important for estimating body burdens and, hence, population-wide risk, as well as for tracking the success of control programs, he said.

Furthermore, they provide a framework for designing epidemiological studies, once they have raised suspicions, says David Kalman, a professor in the Department of Environmental Health at the University of Washington in Seattle. "Knowing in advance the patterns in prevalence [of a problem] gives clues as to how to construct the study."

Studies where subjects are followed after specimens are banked can furnish a clearer picture of how specific compounds influence human health. Once disease has set in, researchers can search the premorbid materials for chemical, biochemical, immunological, and other changes that might predict development of cancer or other diseases.



Peterson Portraits

Human tissue samples provide a measure of dose. Ken Sexton

Kathy J. Helzlsouer, an associate professor of epidemiology in the Department of Epidemiology in the Johns Hopkins University School of Hygiene and Public Health has been conducting studies using banked samples for two decades. In 1974 and again in 1989, over 25,000 blood samples were banked at the Washington County (Maryland) blood bank. Helzlsouer is comparing the level of organochlorines in blood samples from 400 women who have since developed breast cancer with samples from matched controls, although there are no results yet. She is also planning a similar investigation of cadmium in the etiology of a similar number of cases of prostate cancer.

In similar case-controlled studies of serum and urine samples banked since the early 1980s from cohorts in China and Africa, Groopman and his collaborators discovered "a strong interactive effect between aflatoxin exposure and hepatitis B virus exposure in the development of liver cancer." Supporting the hypothesis that aflatoxin had influenced development of cancer, mutations found in the *p53* tumor suppressor gene in these samples were characteristic of types that form uniquely in response to aflatoxin.

Attention has been focused on recent discoveries of genes such as *BRCA1* and *HNPCC*—mutations that are associated with a very high risk of breast and colon cancer, respectively. Researchers have also begun to discover genes that have far more subtle relationships with cancer. For example, "smoking is associated with increased risk of lung, bladder, and other cancers," says Taylor. The protein product of the gene, glutathione-S-transferase MBA (*GSTM1*), binds certain carcinogens, rendering them easier to excrete into the urine. But, Taylor says, "50% of Caucasians, roughly, are walking around with no working copy of that gene. . . . Within two groups who smoke the same amount, the people without the detoxification pathway are at about twice the risk of developing cancer as those with a working copy." Taylor and his colleagues are looking at a whole series of additional genes to try to identify the genetic and environmental determinants of bladder cancer. "We continue to use banked samples and detailed exposure information from the same cases to try to put the genes and exposures together," he said.

In another similarly constructed study using blood samples from the prospective Harvard University Physicians Study, Frederica Perera, a professor of public health in the Division of Environmental Health Sciences at the Columbia University School of Public Health, is investigating the interaction of markers such as polyaromatic hydrocarbon adducts on albumin and DNA with genes controlling metabolism of carcinogens in the development of cancer.

In addition to determining causal agents for specific cancers, researchers can use banked tissues to investigate the molecular pathways by which specific pollutants cause cancer. Several decades ago, Geno Saccomanno, a pathologist at St. Mary's Hospital in Grand Junction, Colorado, noticed that lung cancer was common among uranium miners, who breathe large quantities of radon. Saccomanno began storing blocks of resected lung tumors. Taylor wondered whether the miners' tumors might have different patterns of genetic damage from those of smokers. Examining 52 large and squamous cell cancers from miners, he found that 31% had an identical mutation in the *p53* tumor suppressor gene, but that this mutation was extremely rare in smoking-associated tumors.

Asbestos-associated lung cancers have yet another pattern of mutation. Using a bank of asbestos-associated lung tumors from the Telemark Central Hospital in Norway, Taylor and his colleagues conducted studies, presented at the Meeting of the American Association of Cancer Research in April 1996, showing patterns of base-pair changes that are different from the guanine (G) to thymine (T) mutations that predominate in smoking-associated tumors. Identifying exposure-specific mutations could lead to development of screens for early detection of cancers, and—in the somewhat distant future—to developing chemotherapies, says Taylor.

Different tissues can provide a variety of other insights into the effects of pollution. The U.S. Public Health Service is banking lung specimens from relatively young accident victims in Los Angeles and two control cities, Miami and Seattle, to investigate the effects of ozone on tissue structure, to measure particulates, and possibly to perform other histochemistry and biochemistry studies, says Richard Everson, a medical officer with the PHS.

Taylor praised the study's thorough collection of exposure data, which includes full residential histories of subjects linked to zip code-related pollution measurements taken by the California Air Resources Board, as well as smoking, work-related exposures, and even physical activity levels of subjects.

What to Collect

Organ tissues are unusual in banking studies. Ninety-five percent of materials collected for storage are blood and urine. The reasons are simple. First, it is easy to do cohort studies, because people are used to having both types of samples taken under normal circumstances, says Helzlsouer. The question of what type of sample to use as a control becomes more complicated with, for example, surgical specimens of cancer. "Do you use benign disease such as breast lumps?" asks Helzlsouer.

"We recommend blood [as a standard specimen] because it is readily accessible, and not likely to be contaminated the way hair and nails are," says Bailar. "People wash their hair in all kinds of funny things, and they get all kinds of funny stuff on their hands." DNA can be obtained directly from blood and analyzed using PCR, or white blood cells can be immortalized and frozen in liquid nitrogen for future thawing and expanding.

Even fat can be obtained from blood for sampling of fat-soluble contaminants such as PCBs and pesticides. "There is a very small lipid fraction in the white blood cell that is in equilibrium with whatever is in the blood stream," says Bailar. "The fat fraction of blood should have about the same concentration as any other body fat. Our committee [Committee on National Monitoring of Human Tissues of the National Research Council] did recommend that EPA undertake studies to validate this." Furthermore, "new developments in analytic chemistry over maybe the last 15 years can take this tiny fraction and give you adequate readings of unbelievable sensitivity," says Bailar. Others say that sampling of fat-soluble contaminants can be done still more easily and just as accurately from blood lipids.

Easier than drawing blood is buccal swabs, says Taylor. "It's nothing but a sterile Q-tip. You have the person rub it on the inside of their cheek." In a study investigating contribution of environment and genotype to etiology of cleft lip and palate in Norway, Taylor's colleagues are using buccal swabs to collect samples from controls and from family members. "If we tried to stick a needle into every person that we wanted to get DNA samples from, we would never get close to the participation rates that we can get with buccal swabs," he says. Nonetheless, "blood is still the gold standard because it gives you a lot of DNA that's very high quality."

Urine is also a source of DNA, "because cells are sloughed off from the bladder," says Taylor. "In fact, for bladder cancer, there's increasing interest in using new techniques to look at the DNA of sloughed cells to see if we can detect cancer earlier than using traditional screening techniques."

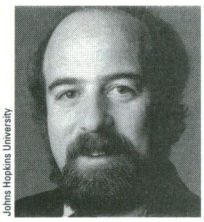
Some materials are much harder to collect than blood and urine, but may offer unique advantages. By virtue of its biological function, the liver accumulates pollutants, but livers can be obtained only from cadavers or surgical specimens. The National Institute of Standards and Technology (NIST) examined how feasible it would be to collect livers from individuals who had been normal and healthy at death, and determined that it was feasible, especially from victims of accidents or gunshots, says Stephen Wise, a research chemist at NIST. Wise was able to collect 300 livers in a single year. According to Wise, lesson number one is that "you need the cooperation of the medical examiner. If the medical examiner is not interested in getting you samples, you won't get any." Of course, samples that are collected from cadavers are inherently biased. "Accident victims don't have the same range of ages and sex as the general population," says Bailar. "They may differ in other ways, too."

Whatever the tissue, researchers should collect it with an eye toward the needs of future investigators, says Taylor. He should know; he has spent years doing research with old samples of cancers from the pathology departments of hospitals. In one study, Taylor says, "I had to go to hospitals all over the country to track down tumors and contact individual pathologists and say, 'Can you find this 30-year-old block?'" Samples are normally fixed in formalin and then embedded in paraffin blocks for storage.

Steve McCaw, Image Associates

The Holy Grail of tissue banking is to relate levels of environmental contamination to health outcomes via doses. Jack Taylor

Old blocks in particular often get hidden in the deepest, darkest corners of a hospital, so retrieving them is no easy task. "We've had them all melted together in a giant blob, or destroyed by fire, so you can't always get what you want," Taylor said. "Getting the pathologist to give up the blocks is often tricky. You have to be careful to return the blocks to the pathologist, something I try hard to do as fast as I can."


Johns Hopkins University

If we don't solve the dilemma of consent, no prospective cohort we design now will be usable. John Groopman

Storing and Maintaining Collections

Storage is the key to longevity of samples, and there are many ways to do it. The best method of storage is to freeze them in liquid nitrogen at temperatures of -180°C to -196°C. "The main reason you would want to use liquid nitrogen is if you are working with any cellular component where you want all metabolic processes to stop dead, and be able to revive the cell," says Elaine Gunter, chief of the NHANES laboratory at the CDC. "Also, if you have a cell that has an infectious agent that you aren't aware of, it keeps the agent from killing the cell." Additionally, liquid nitrogen also prevents the condensation and desiccation that can plague a mechanical freezer, says Gunter. Kalman refers to freezing in liquid nitrogen as a "suspenders and belt" approach that offers the best protection in view of uncertainty about future investigations of stored samples.

On the other hand, if the goal is simply to be able to identify environmental pollutants in tissues at some later date, fairly high temperatures and even room temperatures can be adequate to preserve many of the more common pollutants, such as heavy metals and aromatic hydrocarbons, says Kalman.

For successful long-term freezing, liquid nitrogen is also more of a fail-safe than mechanical freezing, says Elio Riboli, chief of the Unit of Nutrition and Cancer at the International Agency for Research on Cancer (IARC). Pressure differences drive replenishment of the storage containers from a large reservoir of liquid nitrogen, which can stay cold enough to maintain the storage containers for two months without electricity. "I have had samples stored [in mechanical freezers] at -80°C, but we have all had the experience of the freezer breaking down, usually between Saturday night and Sunday," Riboli said.

In the course of a major project conducted by IARC, the European Prospective Investigation into Cancer and Nutrition, samples from 350,000 subjects are being banked. The program is hailed as a model of excellent storage techniques. To avoid having to thaw and refreeze samples every time an analysis is needed, "plasma, serum, buffy coat, and red blood cells will be stored in small plastic straws with a capacity of only 0.5 ml," according to IARC, with 30 ml being collected from each subject.

Pre-labeled jackets in a different color for each of the four blood fractions stored fit tightly around each straw. For insurance, the 28 straws for each subject are divided into identical series of 14, one stored at the center where the blood is collected, and the other at IARC. IARC has developed a system for organizing the samples for quick, easy access. "The large capacity [of liquid nitrogen systems lends itself] to a very organized storage system," says Gunter, "so you can set up a computerized inventory and track by rack, box, position, tray, or whatever."

The CDC has about 500 mechanical freezers storing materials at -70°C, says Gunter. "It's easier where you are going in and out every day." But the CDC is renovating a facility of 220 large-capacity, liquid-nitrogen freezers, large enough to store "literally millions of samples," says Gunter.

As for storage requirements of other materials, the 25,000 samples of sera from the Washington County Blood Bank are stored at -70°C in mechanical freezers over 20 years old, says Helzlsouer. "We still get levels of nutrients and hormones that we would expect, so we think they are fairly stable."

So far, for periods of at least three years, at temperatures of -20°C and -70°C and in alcohol at room temperature, buccal swabs are "pretty stable," says Taylor. "That's the longest we've looked." As for urine, longevity hasn't been established, says Taylor, who freezes most of the samples he collects at -20°C. "I'm just winging it."

NHATS provided some examples of how not to store samples, according to the NRC. Specimens stored in glass bottles sealed with metal caps with foil-lined cardboard inserts had rusty caps, and 10–15% of the specimens contained pieces of foil. Some caps were loose, and specimens had dried out. "Storage artifacts . . . become severe after five to six years of storage," the report concluded.

Informed Consent

Almost ten years have passed since the National Center for Environmental Health (NCEH) of the CDC decided to create a DNA bank from NHANES blood. At the time, says Karen Steinberg, chief of the Molecular Biology Branch of the NCEH, "there weren't a lot of things to test for. All the issues apparent today were not apparent then." But as new gene discoveries began to occur on practically a weekly basis, Steinberg began to worry. "We didn't feel our consent was sufficient to do linked testing," she says.

Groopman says that the debate two years ago over health care reform as it related to preexisting conditions further highlighted the issue of consent. Steinberg is more worried that subjects in research on one gene, who have said they want the results, might be presented by well-meaning researchers with information concerning genes not covered in consent forms, such as breast cancer or colon cancer genes.

These concerns led to a meeting sponsored jointly by the National Center for Human Genome Research and the CDC of medical ethicists, lawyers, and researchers from around the country, and to an article in the 13 December 1995 issue of the *Journal of the American Medical Association* (*JAMA*) by Steinberg and several co-authors. The article advocated policies that some researchers consider to be dangerously restrictive, creating quite a controversy. Such policies include a requirement that it be impossible for anyone, including the principal investigator,


Matthew Gilson

Nationwide surveys are important for estimating population-wide risks and for tracking the success of toxin control programs. John C. Bailar, III

Some Environmental Studies Using Banked Tissues

Name of study	No. of samples	Type of sample	Endpoint
National Human Adipose Tissue Survey (NHATS)	12,000	fat tissues	pesticide residues
National Health and Nutrition Examination Surveys (NHANES)	20,000	blood	mostly nutrition
National Human Exposure Assessment Survey (NHEXAS)	about 950	blood, urine, hair, fingernail	metals, volatile organics, pesticides, PAHs
Breast and prostate cancer studies (Helzlsouer)	25,000	blood	organochlorines and breast cancer, cadmium and prostate cancer
Harvard University Physicians Study	15,000	blood	PAH biomarkers, gene metabolism and carcinogens
Uranium miner studies (Taylor)	52	squamous cell cancers	mutation in the *p53* tumor suppressor gene
Lung cancer study (Taylor)	25	asbestos-associated lung tumors	mutations in smoking-associated tumors
Aflatoxin and liver cancer study (Groopman)	18,000	serum and urine	interaction between aflatoxin and hepatitis B in liver cancer

to link human specimens with individuals, or that a new informed consent must be obtained every time a researcher studies a new gene.

In their rush to protect research subjects, says Caporaso, Steinberg and others are confusing the big risks to individuals of having a *BRCA1* breast cancer gene or *HNPCC* colon cancer gene with risks from metabolic polymorphisms that are insignificant from the standpoint of the individual, yet important to public health. "It would be farfetched to think that [genes for carcinogen-metabolizing proteins] would have health implications for families," says Everson. But the proposed standards would hamstring this research, says Schulte.

The standard of anonymity is impossible to achieve without dumbing information on each individual down to uselessness, some researchers say. It only takes a few pieces of information such as grade and stage of tumor, age at resection, race, and smoking habit to make possible a definitive identity, even if all links in the database have been cut. "As an example of anonymity," says Steinberg, " a set of such parameters should identify at least three people.

"Requiring consent for every new gene to be studied from a sample is untenable," asserts Caporaso, "in that it does not appear to serve either the individual's interest or those of science. The cost is prohibitive. If it's a population-based cohort . . . and you want to write to every person and say we are going to test a genetic marker, this is what it means: with a cohort of 100,000 people, the cost is conservatively in the millions."

But in dealing with human subjects, it is important to err on the side of caution, Steinberg asserts, alluding to the medical establishment's past paternalism. "We are the servants of the public. We have to be very sensitive to issues of importance to the public . . . and we have to deal with their perception of risk."

The *JAMA* authors also recommended "enactment of more general legislation to ensure that no person or institution be able to obtain access, even by court order or subpoena, to either the samples used in research or the specific results of research performed on such samples." Their reasoning was that "although protection may already be provided by certificates of confidentiality, sources are entitled to this higher level of protection in exchange for allowing their samples to be used for research."

The Office for Protection from Research Risks at NIH might develop its own guidance, and the new National Bioethics Advisory Commission being established by President Clinton could issue guidance, says John Miller, the office's deputy director. For the moment at least, such guidance remains the province of individual institutional review boards.

The controversy over informed consent has left those who bank on tissues holding their breath. Tissue banking has proven its value as a versatile method of studying the impact of environmental contamination on human health, and for contributing information for environmental policy-making. Questions such as deciding how to conduct a study, which tissues to collect, and how best to store them can be difficult to answer, but usually yield to creativity, hard work, and money. Researchers fear that current issues surrounding informed consent might not be nearly so tractable. How this controversy plays out will have a profound impact on a crucial area of environmental research.

David Holzman

ERRATA

The second paragraph of the second column on page 23 of the article *The Attack of Asthma* in the January 1996 issue of *EHP* (104:1) mistakenly attributes statistics about Puerto Rican children and asthma to the *New York Times*. The information is taken from an article that appeared in the April 1993 issue of the *American Journal of Public Health* (83:4), entitled Reported Asthma among Puerto Rican, Mexican-American, and Cuban Children, 1982 through 1984. The statistics were also incorrectly identified as the rates of asthma among Puerto Rican, Mexican-American, Cuban, black, and white children between 1982 and1984. They should have been identified as the percentages of children who have ever had asthma. Also in this *EHP* article, the date at the bottom of the first column on page 23 mistakenly reads "between 1976 and 1908." The sentence should read "1980."